Inhibitory Effects of Surfactant Protein A on Surfactant Phospholipid Hydrolysis by Secreted Phospholipases A_2[1]

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Hydrolysis of surfactant phospholipids by secreted phospholipases A_2 ($sPLA_2$) contributes to surfactant dysfunction in acute respiratory distress syndrome. The present study demonstrates that $sPLA_2$-IIA, $sPLA_2$-V, and $sPLA_2$-X efficiently hydrolyze surfactant phospholipids in vitro. In contrast, $sPLA_2$-IIC, -IID, -IIE, and -IIF have no effect. Since purified surfactant protein A (SP-A) has been shown to inhibit $sPLA_2$-IIA activity, we investigated the in vitro effect of SP-A on the other active $sPLA_2$ and the consequences of $sPLA_2$-IIA inhibition by SP-A on surfactant phospholipid hydrolysis. SP-A inhibits $sPLA_2$-X activity, but fails to interfere with that of $sPLA_2$-V. Moreover, in vitro inhibition of $sPLA_2$-IIA-induces surfactant phospholipid hydrolysis correlates with the concentration of SP-A in surfactant. Intratracheal administration of $sPLA_2$-IIA to mice causes hydrolysis of surfactant phosphatidylglycerol. Interestingly, such hydrolysis is significantly higher for SP-A gene-targeted mice, showing the in vivo inhibitory effect of SP-A on $sPLA_2$-IIA activity. Administration of $sPLA_2$-IIA also induces respiratory distress, which is more pronounced in SP-A gene-targeted mice than in wild-type mice. We conclude that SP-A inhibits $sPLA_2$ activity, which may play a protective role by maintaining surfactant integrity during lung injury. ***The Journal of Immunology*****, 2003, 171: 995–1000.**

Acute respiratory distress syndrome (ARDS)[3] is a lung injury with a high mortality rate, characterized by an acute inflammatory response in the lung parenchyma associated with severe injury to the epithelial and endothelial barriers (1). One of the typical features of ARDS is a pronounced alteration of pulmonary surfactant that consists of a complex mixture of phospholipids (80–90%, w/w), neutral lipids (5–10%), and surfactant-specific proteins (5–10%) covering the alveolar surface. The major function of surfactant is to reduce the surface tension at the air-liquid interface of the alveolus. The destruction of surfactant results in loss of alveolar stability and severe deterioration of gas exchange, including alveolar collapse (2).

The role of secreted phospholipases A_2 ($sPLA_2$s) in a variety of inflammatory diseases including ARDS is now clearly established (3, 4). The $sPLA_2$s form a growing family of enzymes that catalyze the hydrolysis of the *sn-2* ester bond of glycerophospholipids, leading to the production of free fatty acids and lysophospholipids that serve as precursors for a variety of lipid-derived mediators involved in numerous biological activities (5, 6). To date, 10 different $sPLA_2$s, referred to as groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, and XII, have been characterized in mammals (7–12). Of particular interest, levels of $sPLA_2$ activity in bronchoalveolar lavage fluids (BALF) of patients with ARDS often correlate positively with the severity of the disease (13). However, the type of $sPLA_2$ involved in this disorder remains unclear. While $sPLA_2$-IIA is able to efficiently hydrolyze surfactant phospholipids, thereby contributing to surfactant alteration as observed in ARDS (14), the involvement of the other cloned $sPLA_2$s in this process is not yet defined, and their roles in ARDS lung disease remain uncertain. In this study we investigated the potential pathological contribution of the newly cloned $sPLA_2$s in ARDS by analyzing their ability to hydrolyze surfactant phospholipids in vitro.

Alteration of surfactant phospholipids occurs very early during the development of ARDS. Therefore, the development of inhibitors of $sPLA_2$ preventing hydrolysis of surfactant phospholipids could represent an auspicious strategy for ARDS treatment. Indeed, despite recent advances in intensive care, the ARDS mortality rate still exceeds 40%, and there is no effective therapy apart from mechanical ventilation and other supportive measures (15). Interestingly, we previously showed that surfactant protein A (SP-A), which is the major surfactant-associated protein, inhibits the in vitro $sPLA_2$-IIA activity through a direct protein-protein interaction (14). SP-A, which is a member of the C-type lectin superfamily and contains a COOH-terminal carbohydrate recognition domain (CRD) (16), plays an important role in pulmonary host defense and seems to inhibit inflammation following lung infection. Decreased SP-A levels in BALF are observed in patients with ARDS or at risk of developing ARDS (17–19). Thus, increased $sPLA_2$ activity associated with decreased SP-A might promote the hydrolysis of pulmonary surfactant observed in ARDS. The aim of the present investigation was also to examine the effects of SP-A on $sPLA_2$ activity in vivo and in vitro.

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[3] Abbreviations used in this paper: ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; CRD, carbohydrate recognition domain; mGIIA $sPLA_2$, group IIA recombinant mouse $sPLA_2$; PC, phosphatidylcholine; Penh, enhanced pause; PG, phosphatidylglycerol; r-GP $sPLA_2$-IIA, recombinant guinea pig $sPLA_2$-IIA; SP-A, surfactant protein A; $sPLA_2$, secreted phospholipase A_2.

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Materials and Methods

Animals and reagents

SP-A and GM-CSF knockout mice (SP-$A^{-/-}$ and GM-$CSF^{-/-}$) were bred from the C57BL/6/129Sv F2 homozygous SP-$A^{-/-}$ and GM-$CSF^{-/-}$ mice as previously described (20, 21). Recombinant guinea pig $sPLA_2$-IIA (r-GP $sPLA_2$-IIA) was produced in our laboratory (22). The recombinant mouse $sPLA_2$s were expressed and purified as described previously (9, 10, 23). The rabbit polyclonal anti-rat SP-A Ab was a gift from F. X. McCormack (University of Cincinnati, Cincinnati, OH). Fatty acid-free BSA, phosphatidylcholine (PC), and phosphatidylglycerol (PG) standards were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of pulmonary surfactant

Animals were killed by i.p. administration of a lethal dose of sodium pentobarbital (Sanofi, Libourne, France). The trachea of mice was cannulated, and BALF collection was performed with a syringe by multiple cycles of instillation and aspiration with unitary 0.5 ml of saline to provide 4 ml of BALF for pulmonary surfactant isolation. Cell-free BALF was obtained after centrifugation ($300 \times g$ for 15 min), and pulmonary surfactant was isolated by centrifugation of cell-free BALF at $19{,}500 \times g$ for 20 min.

SDS-PAGE and immunoblot analysis

SDS-PAGE was performed according to the method described by Laemmli (24). Twelve percent gels were loaded with 5 μg of total pulmonary surfactant phospholipids from the different mice or, as a control, 1 μg of purified human SP-A in the presence of 5% (v/v) 2-ME. Proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and probed with a rabbit polyclonal anti-SP-A Ab (1/50,000). Membrane was then probed with anti-rabbit IgG HRP-linked donkey Ab (1/2,500), washed, incubated with ECL detection reagent (Amersham Pharmacia Biotech, Little Chalfont, U.K.), and exposed to Kodak X-OMAT AR film (Kodak-Pathé, Paris, France). Protein standards (Bio-Rad, Hercules, CA) with molecular masses in the range of 6,500–175,000 were used.

Incubation of $sPLA_2$s with pulmonary surfactant

The $sPLA_2$ assay buffer contained 100 mM Tris, 150 mM NaCl, and 10 mM $CaCl_2$ (pH 8). Surfactant was suspended in this buffer and then submitted to ultrasonication for 5 min (150 W) using an MSE sonifier (Annemasse, France). After adjusting the phospholipid concentration to 2 mM, surfactant was incubated for 1 h at 37°C with the indicated $sPLA_2$s in the presence of 0.1% BSA (final volume, 50 μl). To analyze the role of SP-A in SP-$A^{+/+}$ mice on fatty acid release by r-GP $sPLA_2$-IIA, surfactant (2 mM phospholipid concentration) from SP-$A^{+/+}$ mice was preincubated with 5 μg of rabbit polyclonal anti-SP-A IgG or control rabbit IgG in a final volume of 50 μl overnight at room temperature. Surfactant was then incubated for 1 h at 37°C with 1 μg/ml r-GP $sPLA_2$-IIA, and fatty acid release was analyzed as described below.

Fatty acid analysis

Fatty acids were extracted by Dole's procedure modified by Tsujishita et al. (25) and methylated with diazomethane. The methylated derivatives were separated by gas chromatography on a capillary column containing Supelcowax 10 bonded phase (diameter, 0.32 mm; 30 m long; Supelco, Bellefonte, PA) on a gas chromatograph (5890 series II; Hewlett-Packard, Palo Alto, CA) and detected by mass spectrometry (10-10C; Nermag, Reuil, France).

Analysis of pulmonary surfactant PC and PG

Lipids were extracted from isolated surfactant according to the method described by Bligh and Dyer (26). The separation of phospholipids was achieved by TLC using silica gel plates (Merck, St. Louis, MO) and a water/acetic acid/methanol/chloroform (65/45/3/1, v/v) solvent system. The surfactant PC and PG spots were localized by reference to corresponding standards and extracted from silica gel plates, and then their phosphorus contents were measured according to the method reported by Böttcher et al. (27).

Administration of $sPLA_2$-IIA to mice

SP-$A^{-/-}$ or SP-$A^{+/+}$ 7-wk-old male mice, weighing 25–30 g, were instilled through an intratracheal catheter with either 20 μg of group IIA recombinant mouse $sPLA_2$ (mGIIA $sPLA_2$) dissolved in 50 μl of saline or the same volume of saline containing 5 mM $CaCl_2$. One hour after treatment, mice were killed by an overdose of sodium pentobarbital. BALF was collected, and pulmonary surfactant was isolated as described above. There were no significant differences in the total volume of saline infused into the lungs or in the volume recovered after the lavage procedure among any experimental groups. Mice were cared for in accordance with Pasteur Institute guidelines in compliance with the European animal welfare regulations.

Measurement of respiratory functions

The respiratory function of freely moving mice was measured by barometric plethysmography using whole-body plethysmography (Buxco Electronics, USA) according to the manufacturer's instructions. In brief, anesthetized mice received intratracheal instillation of 20 μg of mGIIA $sPLA_2$ or equivalent volume of saline. Then, each animal was placed in a main chamber and pressure difference between this chamber and a reference chamber were measured with a differential pressure transducer connected to amplifier and recorded with BioSystem XA analyzer software (Buxco Electronics, Birmingham, U.K.). Enhanced pause (Penh) correlated very closely with pulmonary resistance and was calculated as follows: $\text{Penh} = (\text{Te} - \text{Tr})/\text{Tr}(\text{PEP}/\text{PIP})$, where Te is the expiratory time (seconds), Tr is the relaxation time (time of the pressure decay to 36% of total box pressure at expiration), PEP is the peak expiratory pressure (milliliters per second), and PIP is the peak inspiratory pressure (milliliters per second).

Statistical analysis

Results are expressed as the mean $\pm$ SEM for the indicated number of independently performed experiments. Comparisons between values were analyzed by Student's t test for unpaired data, and $p < 0.05$ was considered significant.

Results

In vitro hydrolysis by $sPLA_2$-IIA of different natural surfactants containing various amounts of SP-A

We previously demonstrated the inhibitory effect of SP-A on $sPLA_2$-IIA activity on synthetic PG substrates in vitro (14). To assess the relationship between SP-A and surfactant phospholipid hydrolysis by $sPLA_2$-IIA, surfactants purified from wild-type mice, mice deficient in SP-A (SP-$A^{-/-}$ mice), or mice with increased alveolar SP-A (GM-$CSF^{-/-}$ mice) were used as substrates. In GM-$CSF^{-/-}$ mice, the increased SP-A content results from an alveolar accumulation of surfactant-associated proteins and phospholipids caused by impaired clearance by alveolar macrophages (28). The phospholipid concentrations of the different surfactants from SP-$A^{+/+}$, SP-$A^{-/-}$, and GM-$CSF^{-/-}$ mice were adjusted to 2 mM, and the concentration of SP-A was estimated by immunoblotting. As expected, SP-A was undetectable in the pulmonary surfactant from SP-$A^{-/-}$ mice, and its level was considerably increased in GM-$CSF^{-/-}$ mice compared with SP-$A^{+/+}$ mice (Fig. 1*A*).

The $sPLA_2$-IIA activity was assessed by measuring the release of free fatty acids generated by surfactant phospholipid hydrolysis. As shown in Fig. 1*B*, the release of fatty acids by $sPLA_2$-IIA was more pronounced in SP-$A^{-/-}$ mice than in SP-$A^{+/+}$ mice and was quasi-nonexistent in GM-$CSF^{-/-}$ mice. Previous studies have shown that neither the levels of surfactant proteins B, C, and D nor the sizes of alveolar phospholipid pool and their compositions are altered in SP-$A^{-/-}$ mice (20). Furthermore, SP-$A^{-/-}$ mice have no discernable abnormalities in surface activity or film stability even when lung injury is induced (20, 29). In GM-$CSF^{-/-}$ mice the surfactant phospholipid composition is also unaltered. Therefore, our results suggest that surfactant hydrolysis by $sPLA_2$-IIA is inversely correlated to the levels of the surfactant-associated SP-A protein.

Although the targeted disruption of the murine SP-A gene does not affect the level and composition of surfactant phospholipid (20), we aimed to confirm whether the increase in fatty acid release by $sPLA_2$-IIA was indeed due to the absence of SP-A rather than to any potential difference in the properties of surfactant of SP-$A^{-/-}$ mice. Thus, SP-$A^{+/+}$ surfactant was incubated overnight at

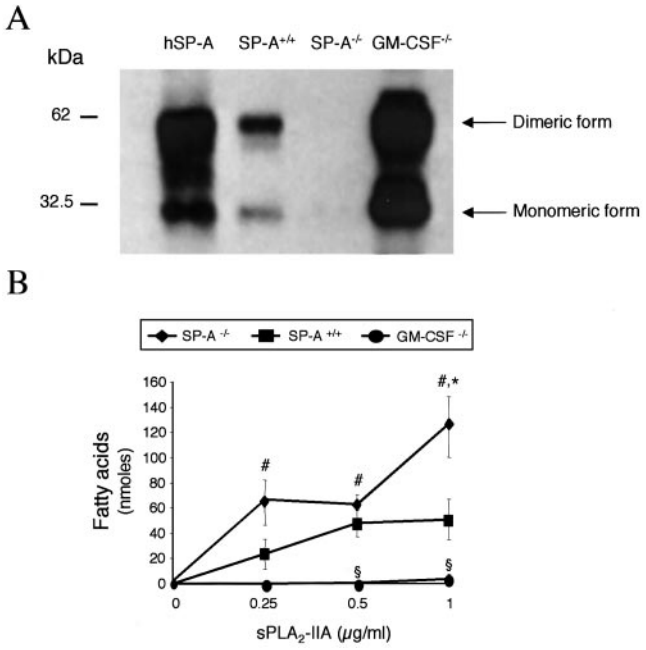

FIGURE 1. In vitro analysis of the hydrolysis by $sPLA_2$-IIA of different natural surfactants containing various amounts of SP-A. *A*, Analysis by immunoblotting of surfactant-associated SP-A content in SP-$A^{-/-}$, SP-$A^{+/+}$, and GM-$CSF^{-/-}$ mice was performed after adjusting the phospholipid concentration to 2 mM. *B*, Fatty acid release was measured after 1-h incubation of $sPLA_2$-IIA with 2 mM surfactant phospholipids from each mouse genotype. Results are expressed as the mean ± SEM obtained from three separate experiments, each being performed on a pool of surfactants collected from untreated mice. *, $p < 0.05$, SP-$A^{-/-}$ mice vs SP-$A^{+/+}$ mice; #, $p < 0.02$, SP-$A^{-/-}$ mice vs GM-$CSF^{-/-}$ mice; §, $p < 0.05$, GM-$CSF^{-/-}$ mice vs SP-$A^{+/+}$ mice.

room temperature with a polyclonal anti-SP-A Ab to neutralize SP-A; $sPLA_2$-IIA (1 μg/ml) was added for 1 h at 37°C, and the release of fatty acids was measured. Hydrolysis of surfactant phospholipids increased significantly when the surfactant was preincubated with the anti-SP-A Ab, confirming the inhibitory effect of SP-A on $sPLA_2$-IIA activity (Fig. 2).

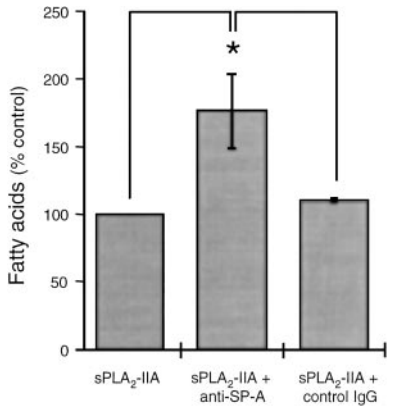

FIGURE 2. Neutralization by anti-SP-A Ab of the inhibitory effect of surfactant-associated SP-A on $sPLA_2$-IIA activity. Surfactant from SP-$A^{+/+}$ mice (2 mM phospholipid concentration) was preincubated overnight at room temperature with anti-SP-A IgG or nonrelevant IgG; $sPLA_2$-IIA (1 μg/ml) was then added for 1 h at 37°C, and the release of fatty acid was measured as indicated in *Materials and Methods*. Results are expressed as the mean ± SEM percentage of the control value ($n = 3$). *, $p < 0.05$.

Analysis of in vitro surfactant hydrolysis by various $sPLA_2$s and effect of SP-A on $sPLA_2$ activity

Recently, novel $sPLA_2$s have been cloned, but their biological roles remain unknown (6). In this study we tested in vitro their ability to hydrolyze surfactant phospholipids using surfactant from SP-$A^{-/-}$ mice (Fig. 3). As previously found (14), $sPLA_2$-IIA hydrolyzed surfactant phospholipids, and PG was the most preferred substrate for this enzyme. By contrast, the other type II $sPLA_2$s, including IIC, IID, IIE, and IIF enzymes, were ineffective in hydrolysis. Both $sPLA_2$-V and $sPLA_2$-X hydrolyzed PC and PG. In summary, only $sPLA_2$-IIA, $sPLA_2$-V, and $sPLA_2$-X hydrolyzed surfactant phospholipids.

We next investigated whether the presence of surfactant-associated SP-A interferes with PC and PG hydrolysis by comparing the activities of $sPLA_2$-IIA, $sPLA_2$-V, and $sPLA_2$-X on surfactants from SP-$A^{+/+}$ and SP-$A^{-/-}$ mice. There was no significant difference in the hydrolysis of surfactant phospholipids from SP-$A^{+/+}$ and SP-$A^{-/-}$ mice by $sPLA_2$-V (Fig. 4). In contrast, surfactant phospholipids from SP-$A^{-/-}$ mice were significantly more susceptible to hydrolysis by $sPLA_2$-IIA and $sPLA_2$-X than those from wild-type mice. The selective inhibition of $sPLA_2$ by SP-A (SP-A has no effect on $sPLA_2$-V) and our previous observation of a direct binding between SP-A and $sPLA_2$-IIA (14) strongly suggest that the effect of SP-A is due to a direct interaction with the enzyme and not to an alteration of phospholipid structure.

In vivo effect of SP-A on surfactant hydrolysis by $sPLA_2$-IIA

Our above in vitro studies showed that $sPLA_2$-IIA hydrolyzed surfactant phospholipids and that SP-A reduced susceptibility of surfactant to hydrolysis. However, $sPLA_2$-IIA activity depends not only on the chemical nature of phospholipids, but also on their physical state (30). For instance, $sPLA_2$-IIA is very active toward micellar lipids as in our in vitro assay (31). Because surfactant phospholipids form in vivo a monolayer film that may be differentially hydrolyzed by $sPLA_2$-IIA, we examined the ability of $sPLA_2$-IIA to hydrolyze surfactant phospholipids in vivo in SP-$A^{-/-}$ and SP-$A^{+/+}$ mice. In these experiments mGIIA $sPLA_2$ was instilled intratracheally into the lungs of SP-$A^{-/-}$ and SP-$A^{+/+}$ mice, and surfactant phospholipid hydrolysis was analyzed (Fig. 5). The ratio of PC/PG increased following the administration of $sPLA_2$-IIA for SP-$A^{-/-}$ mice (Fig. 5*A*). While no change in PC

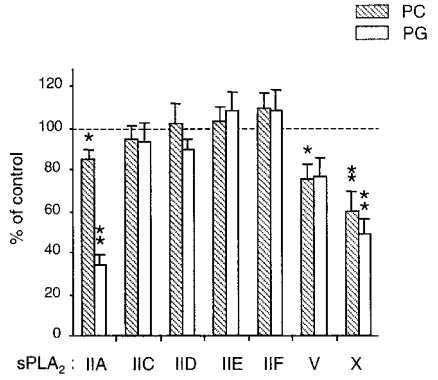

FIGURE 3. Analysis of in vitro surfactant hydrolysis by various $sPLA_2$s. Surfactant (2 mM phospholipid concentration) from SP-$A^{-/-}$ mice was incubated for 1 h at 37°C with 1 μg/ml of the indicated $sPLA_2$. After extraction of surfactant phospholipids, PC and PG were separated, and their amounts were measured as described in *Materials and Methods*. Data are expressed as a percentage of the control value and are the mean ± SEM of five independent experiments. *, $p < 0.03$; **, $p < 0.006$ (compared with control).

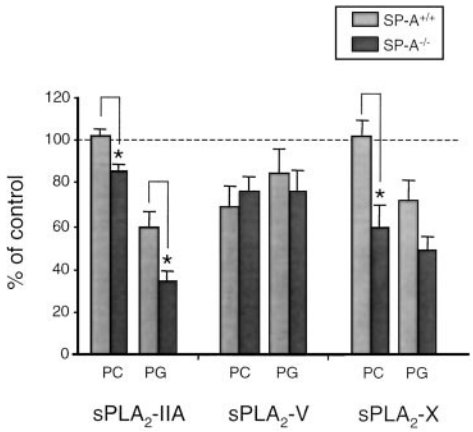

FIGURE 4. In vitro effect of surfactant-associated SP-A on the activities of $sPLA_2$-IIA, $sPLA_2$-V, and $sPLA_2$-X. Following the protocol indicated in Fig. 3, surfactant (2 mM phospholipid concentration) from SP-$A^{-/-}$ or SP-$A^{+/+}$ mice was incubated for 1 h at 37°C with 1 μg/ml of the indicated $sPLA_2$. Data are expressed as a percentage of the control value and are the mean ± SEM of five independent experiments. *, $p < 0.04$, SP-$A^{-/-}$ mice vs SP-$A^{+/+}$ mice.

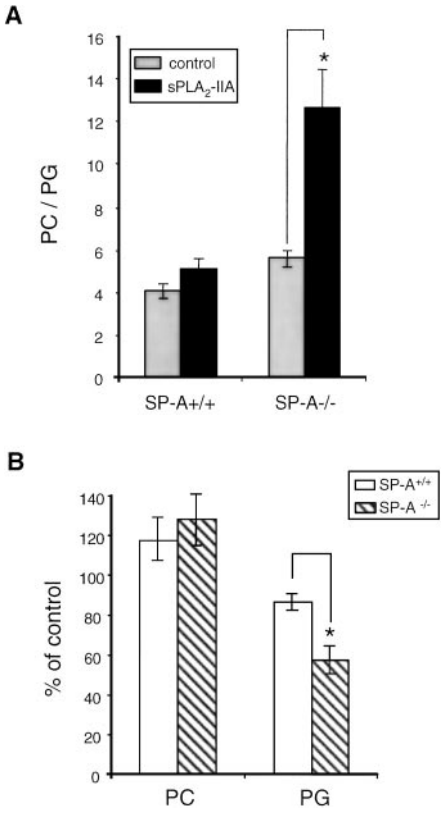

FIGURE 5. In vivo effect of surfactant-associated SP-A on surfactant phospholipids hydrolysis by $sPLA_2$-IIA. BALF were obtained 1 h after intratracheal instillation of saline or 20 μg of mGIIA $sPLA_2$. Surfactants were then isolated and analyzed as indicated in *Materials and Methods*. *A*, Effect of mGIIA $sPLA_2$ on the ratio of PC to PG in the surfactant from SP-$A^{+/+}$ and SP-$A^{-/-}$ mice. *B*, Comparison of the amounts of surfactant PC and PG between SP-$A^{+/+}$ and SP-$A^{-/-}$ mice treated with mGIIA $sPLA_2$, expressed as a percentage of the control value (in corresponding saline-treated mice). All results are the mean ± SEM obtained from five animals. *, $p < 0.007$.

levels was observed following treatment with $sPLA_2$-IIA, the PG content decreased significantly (13.5 ± 4 and 38.4 ± 7% for SP-$A^{+/+}$ and SP-$A^{-/-}$ mice, respectively, compared with corresponding control animals that were treated with saline (Fig. 5*B*). These data indicate that $sPLA_2$-IIA is able to efficiently hydrolyze PG under in vivo conditions. Secondly, we observed that the surfactant PG content decreased markedly in SP-$A^{-/-}$ mice compared with wild-type mice after administration of $sPLA_2$-IIA. Collectively, these results demonstrate that surfactant-associated SP-A reduces the hydrolysis of surfactant PG by $sPLA_2$-IIA in vivo.

In vivo impairment of surfactant function by $sPLA_2$-IIA and protective effect of SP-A

To determine whether the decrease in PG leads to a defect of the surfactant function, we examined the effect of intratracheal administration of mGIIA $sPLA_2$ on respiratory functions expressed as an increase in Penh. The latter is an index of airway resistance resulting from lung injury. In $sPLA_2$-IIA-treated mice, Penh increased after 3 h, peaked within ~4 h, and then returned to basal levels by 6 h (data not shown). The increase in lung resistance was significantly higher in SP-$A^{-/-}$ mice than in wild-type mice. These results suggest that PG hydrolysis by $sPLA_2$-IIA leads to an impairment of lung surfactant function, and that SP-A is effective in protecting the lung from injury induced by mouse $sPLA_2$-IIA.

Discussion

Clinical studies have suggested that $sPLA_2$s play a role in the pathogenesis of ARDS. Elevated levels of $sPLA_2$ activity have been reported in the BALF of patients; moreover, these levels correlated with the severity of the disease (13). However, the type of $sPLA_2$s involved in ARDS has not been clearly established. Furthermore, the levels of SP-A, a surfactant protein with known in vitro inhibitory capacity of $sPLA_2$-IIA activity, are considerably decreased in patients with ARDS. These considerations prompted us to investigate 1) the ability of newly cloned $sPLA_2$ family members to hydrolyze surfactant phospholipids, and 2) the potential effect of surfactant-associated SP-A on the activities of these various $sPLA_2$s.

This study shows that the release of fatty acid, resulting from in vitro surfactant hydrolysis by $sPLA_2$-IIA, is inversely correlated with the level of SP-A present in pulmonary surfactant (Fig. 1). Moreover, when SP-A present in surfactant from wild-type mice is neutralized with an anti-SP-A Ab, a significant increase in the release of fatty acids is observed (Fig. 2). Taken together, these data suggest that the absence of SP-A exacerbates the susceptibility of surfactant to degradation by $sPLA_2$-IIA.

Mammalian $sPLA_2$s are distinguished by their structural and enzymatic properties (6, 23). Subtle differences in their sequences markedly affect their substrate interfacial binding and rate of hydrolysis (32, 33). Here we analyzed the efficiencies of various $sPLA_2$s, including recently cloned $sPLA_2$s, to hydrolyze surfactant. Although most of these enzymes are expressed in the lung (6, 10), their enzymatic activities toward surfactant phospholipids have never been investigated. This present study shows that in addition to $sPLA_2$-IIA, $sPLA_2$-V and $sPLA_2$-X are able to hydrolyze in vitro surfactant phospholipids. In these conditions PG is the most preferred substrate for $sPLA_2$-IIA, while $sPLA_2$-V and $sPLA_2$-X hydrolyze PC and PG with similar rates (Fig. 3). By contrast, the other analyzed $sPLA_2$s (IIC, IID, IIE, and IIF) are inefficient in inducing surfactant phospholipid hydrolysis.

When comparing the hydrolysis of surfactant phospholipids from SP-$A^{-/-}$ and SP-$A^{+/+}$ mice, we observed that surfactant-associated SP-A could inhibit $sPLA_2$-IIA and $sPLA_2$-X activities, but failed to interfere with that of $sPLA_2$-V (Fig. 4). These findings are of great interest because it has been shown that $sPLA_2$-IIA and

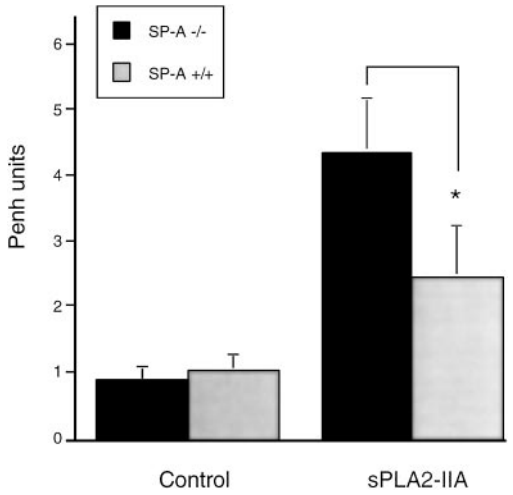

FIGURE 6. Effect of sPlA$_2$-IIA instillation on respiratory function of SP-A$^{+/+}$ and SP-A$^{-/-}$ mice. mGIIA sPLA$_2$ (20 μg) or an equivalent volume of saline was administered to anesthetized mice as indicated in Fig. 5. Each animal was placed in a separate chamber of the plethysmograph, and Penh was determined as detailed in *Materials and Methods*. The figure shows the Penh increase measured 4 h after mGIIA sPLA$_2$ or saline instillation and expressed as the mean ± SEM results obtained from three animals. *, $p < 0.05$.

sPLA$_2$-X bind to the CRD domain of the M-type receptor (34–36), and a structurally similar CRD domain is also present in SP-A (37). Moreover, the binding of sPLA$_2$ to this receptor leads to an inhibition of catalytic activity (38). Surfactant-associated SP-A may therefore act as an endogenous inhibitor of sPLA$_2$-IIA and sPLA$_2$-X by interacting with these sPLA$_2$s through its CRD domain.

Our results may have important pathophysiological relevance, as patients with ARDS have elevated sPLA$_2$ activity and decreased SP-A concentrations in their BALF (17–19). However, no direct causal relationship has been demonstrated between the alteration in SP-A levels and the acute destruction of surfactant phospholipids. This led us to investigate the physiological consequence of the inhibition of sPLA$_2$-IIA activity by surfactant-associated SP-A under in vivo conditions. We first checked whether mouse sPLA$_2$-IIA was able to hydrolyze surfactant phospholipids under in vivo conditions in mice. Several studies have shown that intratracheal administration of sPLA$_2$s can induce lung injury with interstitial and alveolar edema, accumulation of inflammatory cells, and free fatty acid release (14, 39, 40), which are pathological features typical of those seen in the lungs of ARDS patients. However, these studies have often used heterologous sPLA$_2$s from snake venoms, which have much higher ability than mammalian sPLA$_2$s to hydrolyze phospholipids on packed monolayer structures. Moreover, none of these studies used mice as an animal model. In the present study no hydrolysis of surfactant PC was observed in sPLA$_2$-IIA-treated mice. This is not surprising, since sPLA$_2$-IIA, which bears basic residues resulting in positive charges of its interfacial binding surface, cannot bind well to PC vesicles, as described for PG (33, 41, 42).

In contrast to PC, we observed a marked decrease in surfactant PG, particularly in sPLA$_2$-IIA-treated SP-A$^{-/-}$ mice. This finding is consistent with findings that sPLA$_2$-IIA is >100-fold more active on anionic, rather than zwitterionic, phospholipids (9, 30). Remarkably, hydrolysis of PG occurs as early as 1 h after sPLA$_2$-IIA administration, indicating that this enzyme could account for surfactant abnormalities observed at early phases in ARDS (17). In fact, alterations in the relative composition of the surfactant phospholipids are seen in patients with ARDS (17, 18, 43, 44). A decrease in PG levels (by >80% in three of these studies) and a moderate reduction of PC levels were observed. Taken together, these data support a role for sPLA$_2$-IIA in the alterations in PG levels occurring during the early phase of ARDS.

Therefore, it should be of great interest to know whether PG hydrolysis contributes to the pathophysiologic events encountered in ARDS. Surfactant from sPLA$_2$-IIA-treated SP-A$^{-/-}$ mice showed an increase in the PC/PG ratio (Fig. 5*A*). Interestingly, Hite et al. (45) observed PG hydrolysis by sPLA$_2$ in asthmatic patients. They demonstrated a positive correlation between the PC/PG ratio and surfactant dysfunction, and they concluded that the hydrolysis of surfactant PG by sPLA$_2$ may be a mechanism contributing to surfactant dysfunction. Moreover, we showed here that intratracheal administration of mouse sPLA$_2$-IIA leads to a time-dependent increase in Penh, demonstrating that the enzyme has a deleterious effect on lung function (Fig. 6). In this study we did not demonstrate a direct relationship between the decrease in PG content and the impairment of lung function. However, as PG has a relatively high content of unsaturated fatty acids, it may be functionally important because of its capacity to increase monolayer film fluidity and adsorption (46). It has also been shown that PG prevents the collapse of the surfactant film (47). Thus, surfactant PG hydrolysis by sPLA$_2$-IIA may contribute to surfactant dysfunction. The pathophysiological relevance of these results is linked to the fact that the surface activity of the surfactant film from patients with ARDS is abnormal, including a reduced rate of absorption, higher surface compressibility, and lower film stability index (17, 48).

Surfactant PG from SP-A$^{-/-}$ mice was significantly more susceptible to hydrolysis by sPLA$_2$-IIA compared with that from wild-type mice (Fig. 5*B*). This pronounced susceptibility was not due to differences in surfactant composition, since mice lacking SP-A produce similar levels of surfactant phospholipids and of other surfactant proteins compared with wild-type mice (20). We also observed that the increase in Penh resulting from sPLA$_2$-IIA administration was higher in SP-A$^{-/-}$ mice compared with wild-type mice. Taken together, these data strongly suggest that SP-A is an efficient endogenous inhibitor of sPLA$_2$-IIA enzymatic activity and thereby plays a protective role in maintaining pulmonary surfactant integrity during lung injury. Whether endogenous SP-A can also protect surfactant from hydrolysis by other sPLA$_2$s that might be overexpressed during mouse acute lung injury remains to be determined.

This report also demonstrates a deleterious effect of sPLA$_2$-IIA, sPLA$_2$-V, and sPLA$_2$-X on pulmonary surfactant, suggesting a possible involvement of these enzymes in the pathogenesis of ARDS. Our findings support a potential role for SP-A in the protection of surfactant in the early phases of ARDS. Our results corroborate the view that surfactant replacement therapy for ARDS might be enhanced by addition of SP-A.

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